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Effect of an exo-polysaccharide from the culture broth of *Hericium erinaceus* on enhancement of growth and differentiation of rat adrenal nerve cells

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Abstract

It was found that an exo-biopolymer (M.W. 1,000,000, molar ratio of 1.5 : 1.7 : 1.2 : 0.6 : 0.9, glucose : galactose : xylose : mannose : fructose, purity 99%) purified from the liquid culture broth of *Hericium erinaceus* mycelium enhanced the growth of rat adrenal nerve cells. The polymer also improved the extension of the neurites of PC12 cell. Its efficacy was found to be higher than those from known nerve growth factors such as Nerve Growth Factor (NGF) and Brain-Derived Nerve Factor (BDNF). The effect of two standards has not been observed above 0.1 (mg l^{-1}) of supplementation; however, the polymer did show the effect of cell growth and neurite extension at up to 1.0 (mg l^{-1}) of addition. While the polymer improved both cell growth and neurite extension, NGF and BDNF did only outgrowth of the neurites. Maximum cell density and length of the neurites were observed as 1.5×10^5 (viable cells ml^{-1}) and 230 μm, respectively in adding 0.8 (mg l^{-1}) of the biopolymer for 8 days cultivation. The control growth was observed only as 1.2×10^5 (viable cell ml^{-1}) of maximum cell density and 140 μm of maximum length, respectively. It was also confirmed that the polymer reacted with the nerve cells within 30 min after adding the sample, compared to 80 min in adding two other growth factors. Number of neurite-bearing cells remained relatively steady in adding the polymer even when the cell growth started to be decreased. It was interesting that the polymer effectively delayed apoptosis of PC12 cells by dramatically reducing the ratio of apoptotic cells to 20% from 50% of the control.

Introduction

There have been great outcomes about *in vitro* cultivation of several kinds of nerve cells including human nerve embyronic stem cells (Brown 1981; Willis and Coggeshall 1991; deSouza et al. 1995). It can definitely increases the possibility of treating nerve related diseases, such as Alzheimer's and Parkison's diseases and others (Schorderet 1995; Brinaga 1994; Brown 1981). However, there have been still many limitations in cultivating mammalian nerve cells due to low proliferation and differentiation of the neurites compared to other cell lines even though functions of several neutrophic factors like Brain-Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neurotropins-3 (NT3) have been revealed recently (Thoenen et al. 1978; Hokfelt 1991; Patterson and Nawa 1993). The studies on their applications are just beginning (Thoenen and Barde 1980; Barde et al. 1982; Fantl et al. 1993; Faden and Salzam 1992) because the growth mechanisms of nerve cells are not clearly understood yet as well as the relation between neurites and neurotransmit systems.

It has been proved that many nerve cells required additional growth factors, such as NGF, BDNF and epithelial cells, etc. for *in vitro* cultivation (Cunha et

al. 1990; Wang et al. 1990; Frank and Westerfield 1983; Silos-Santiago and Snider 1992; Roush 1996; Kaplan et al. 1993; Merlino et al. 1993; Kumar et al. 1990). It was also found that some tumor cells display more differentiated features and contain cells resembling Schwann cells of the glial lineage, of secretory cells expressing markers of neuroendocrine differentiation (Cooper et al. 1990; Greene and Tischler 1976). Moreover, many chemicals and biological response modifiers are known to promote *in vitro* morphological, biochemical, and ultrastructural changes to well-differentiated neuroelectrodermal phenotypes (Abemayor and Sidell 1989). Lectin and other chemicals from natural resources also demonstrated the ability of affecting the growth of nerve cells (Kawagish et al. 1993, 1994). Mushroom can be a good candidate to play a role in inducing neuronal differentiation and promoting neuronal survival (Wasser and Weis 1997; Shibata et al. 1989; Mizuno 1995; Eisenhut et al. 1995). Among them, *Hericium erinaceus* is an widely distributed edible mushroom, saprophytically inhabiting on dead trunks of hardwoods such as oak, beech and Japanese walnut, etc. It has been reported that fruiting body and mycelium of *H. erinaceus* could work on nerve systems in *in vitro* experiments (Aronone et al. 1994; Kuwahara et al. 1992; Kimura et al. 1991; Mizuno 1995; Liu et al. 1999). Some of them demonstrated the stimulation of NGF synthesis for treating neuronal disorders such as Alzheimer's disease and peripheral nerve regeneration (Ayer et al. 1978; Furukawa et al. 1987; Kawagish et al. 1994). Therefore, in this work, the effects of an exo-polysacharide isolated from *H. erinaceus* on the growth of rat pheochromocytoma cells (PC12) are to be investigated since PC12 cells are most widely used to be an *in vitro* model of neuronal differentiation.

Materials and methods

Preparation of sample

The crude sample was collected from submerged culture broth of *Hericium erinaceus* mycelium after 12 days fermentation (pH 4.5, 28 ℃, 120 rpm in a rotary shaker). Detail culture method was described elsewhere (Lee et al. 1996). The culture broth was centrifuged at 200 g to remove the mycelium in the broth. The supernatant was dialyzed by a 200,000 MW cut-off membrane and centrifuged at 500 g for 30 min, then precipitated by adding two volume of acetone. The precipitate was resolved in distilled water for 24 h at room temperature. This solution was centrifuged again at 500 g for 30 min. The precipitate was dissolved in distilled water and 0.1 N NaOH, then separated by ion exchange chromatogaraph (DEAE celluose resin, Pharmacia, Cambridge, USA) with 2 M NaCl elution solution and followed by Sephadex (CL-5B, pharmacia, Cambridge, USA) chromatography. The purified fraction was trimethysilyationized and analyzed by Gas chromatography (Varian, Star 3400, Campbell, USA) and prep-HPLC (Waters, Chicago, USA) (Brobst and Lott 1996; Lee and Kang 1999). The sample was found to be an exo-polysaccharide having molar ratio of 1.5 : 1.7 : 1.2 : 0.6 : 0.9 (glucose : galactose : xylose : mannose : fructose) and its molecular weight was determined as 1,000,000 dalton (purity 99%) (Lee and Kang 1999). The purified sample was sterilized them through 0.2 μm filter paper before adding into the culture.

Cell culture and biochemical analysis

Rat pheochromocytoma nerve cell, PC12 (ATCC, Rockville, USA) was grown in DMEM/F12 basal medium containing 365 mg l^{-1} of L-glutamine (GIBCO, Grand Island, USA) and 40 μg ml^{-1} of gentamycine enriched with 10% FBS (GIBCO, Grand Island, USA) in a 37 ℃ CO_2 incubator supplying 5% CO_2 in air. The growth of PC12 cell in adding various concentrations of exo-polysaccharide purified from *H. erinaceus* culture broth or two standards, BDNF and NGF (Genzyme, Cambridge, USA) was estimated by the trypan blue dye exclusion method everyday (Frehney 1983). The effect of sample concentration on the growth of PC12 cells was observed by adding various concentrations of the sample into the culture medium. The neurite outgrowth of PC12 cell was monitored under an inverted microscope with a graticule (Rukenstein and Greene 1983) as a measure of differentiation of nerve cells. The ratio of neurite bearing cells to total cells was also calculated by counting total and neurite bearing cells in a haemacytometer. Apoptosis of the cells was measured as follows: The cells cultured with samples were mixed with 4 μl of a dye solution which contains acridine orange and 100 μl of ethidium bromide (1 : 1 v/v) according to cultivation time. The size and numbers of the stained cells were counted through a fluorescent microscope and from pictures were compared to those of normal cells, then the ratio of

apoptotic cells to total cells was estimated by counting the stained cells (Frehney 1983).

The acidification kinetics of the cells responding to samples was observed by a microphysiometer (Molecular Device, New Brunswick, USA). In tests for chemotherapeutic efficacy and toxicity, acidification rate has been used as the cell activity and viability indicator (Wada et al. 1992). The microphysiometer is designed to rapidly monitor the cell growth and biological response by measuring pH changes within the cells, compared to the control which was not treated with samples as follows (Longchuan and Armen 1999; Masanori and Hiroshi 1997): $3\times 10^5 ml^{-1}$ of the cells were put into a capsule cup in the microphysiometer and incubated for 24 hours at 37 °C. Running buffer (PBS buffer) and culture medium (DMEM/F12 with 10% FBS) was continuously flown into the capsule, and then pH was measured pH changes in the capsule according to time (Longchuan and Armen 1999).

All of the experiments were carried out at least five duplications. The data points in Tables and Figures are the mean of five duplicated experiments and the bar is the standard mean deviation calculated by Statistical Analysis System (SAS, NC, USA).

Results and discussion

Figure 1–3 show the effect of a purified exo-polymer and two standards, BDNF and NGF on the growth of PC12 cells. The cell growth was more greatly enhanced in adding the exo-polymer than in adding the standards; 1.51×10^5 (viable cells ml^{-1}) of maximum cell density at 0.8 (mg l^{-1}) of biopolymer addition vs. 1.2×10^5 (viable cells ml^{-1}) at 0.1 (mg l^{-1}) of BDNF or NGF addition. While only 1.0×10^5 (viable cell ml^{-1}) of maximum cell density was maintained without any supplementation (the control case). In general, both nerve growth factors also improved the growth of PC12 cells in adding 0.1 (mg l^{-1}) of the highest supplementation for maintaining high efficacy, but not remarkably, compared to that in adding the exo-polymer. At the above of 0.8 (mg l^{-1}) of biopolymer addition, the enhancement of cell growth was not observed. It tells that there must be an optimal addition concentration to improve the cell growth, showing in the range of 0.8 (mg l^{-1}) to 1.0 (mg l^{-1}). It may be possibly due to the limitation of solubility of the sample at high addition into the culture liquid. It was also found that both standards did not significantly improve the cell growth in adding more than 0.1 (mg l^{-1}). For most cases, the growth of PC12 cells was improved as the addition concentration of the sample or the standards was increased; however, there was not much difference observed in enhancing the cell growth in adding two nerve growth factors. It was interesting that the polymer could prevent sudden drop of cell growth at latter period of the cultivation but others could not. However, it is evident that the sample and nerve growth factors can improve the growth of PC12 cells and maintain relatively higher cell concentration than the case without supplementation. It can also by supported by the results that the compounds from *H. erinaceus* enhanced the synthesis of nerve growth factor, NGF from 70 pg ml^{-1} to 175 pg ml^{-1} (Furukawa et al. 1987; Kawagish et al. 1994).

Figure 4 shows the kinetics of responding to target cells by adding the effectors. The acidification rate was not much changed without adding the effectors within four hours, which was considered to be the control. The cell growth was greatly increased in adding the sample, up to 130 % of the control within 30 min, then remained relatively steady. Both NGF and BDNF also enhanced the cell growth up to ca. 110 %, but not much than the sample. Their response time was even longer than the exo-polymer, such as 85 min for nerve growth factors vs. 30 min for the polymer, respectively. It tells that the polymer can

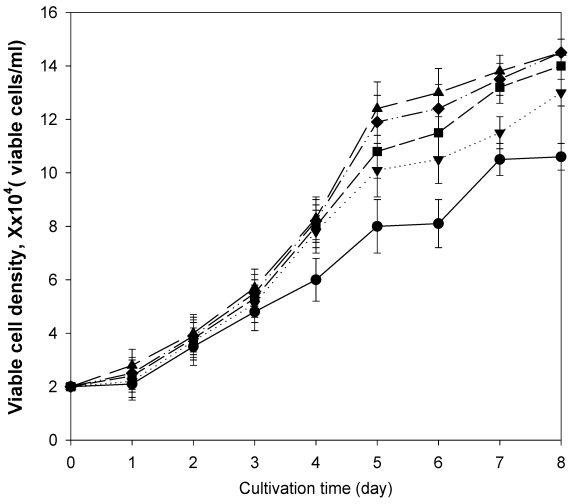

Figure 1. The effect of a biopolymer from *H. erinaceus* on the growth of PC12 cells as a function of supplementation concentration: Control, no addition (●), 0.2 mg l^{-1} (▼), 0.5 mg l^{-1} (■), 0.8 mg l^{-1} (♦), 1.0 mg l^{-1} (▲).

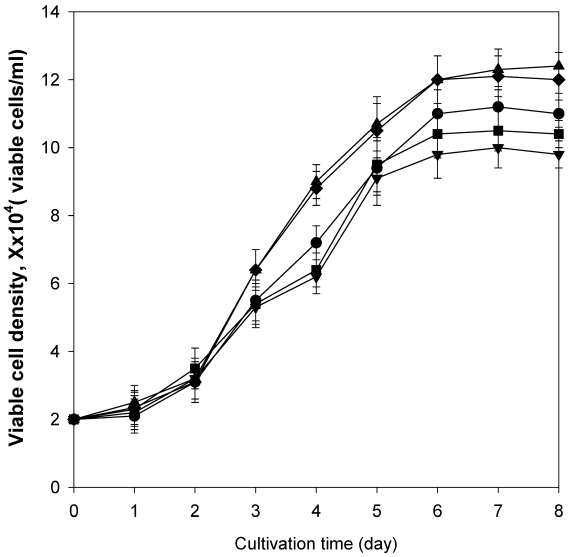

Figure 2. The effect of BDNF on the growth of PC12 cells as a function of supplementation concentration: Control (●), 0.02 mg l^{-1}(▼), 0.05 mg l^{-1} (■), 0.01 mg l^{-1} (♦), 0.12 mg l^{-1} (▲).

react with the cells much faster than two standards and greatly increase the cell growth. Similar result was illustrated in Figure 1, and it is evident that the exo-polymer can increase the growth of PC12 cells within 30–40 min after being supplemented.

Figure 5 proves that the polymer does not affect only the growth of PC12 cells but also the extension of the neurites of nerve cells. For the control, the

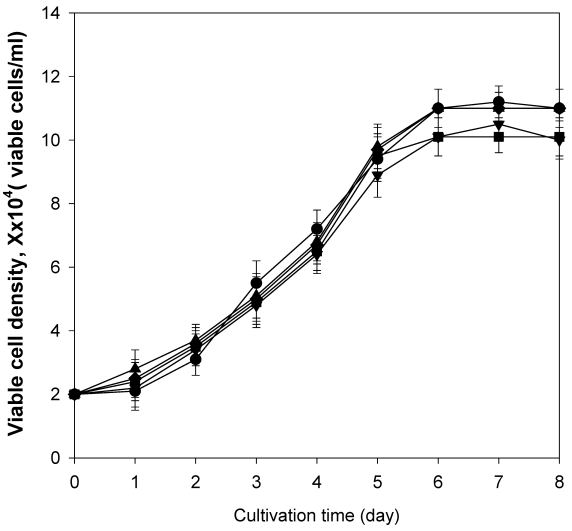

Figure 3. The effect of NGF on the growth of PC12 cells as a function of supplementation concentration: Control (●), 0.02 mg l^{-1} (▼), 0.05 mg l^{-1} (■), 0.01 mg l^{-1} (♦), 0.12 mg l^{-1} (▲).

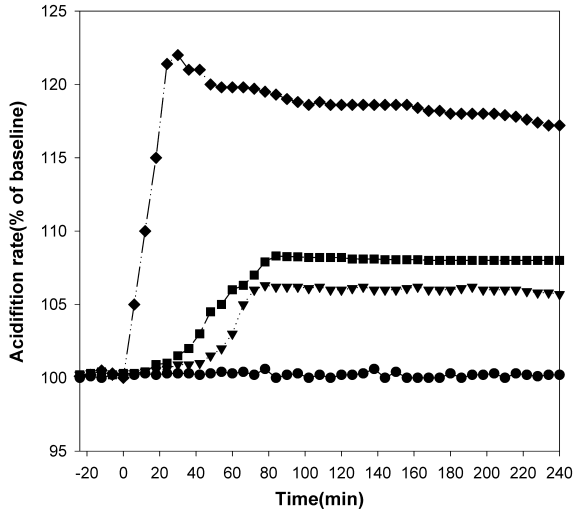

Figure 4. The result of measuring cellular activity of PC12 cells by adding several nerve growth factors for 4 h, using a microphysiometer: Biopolymer(♦), BDNF(■), NGF(▼), Control(●).

neurites did not grow any longer after four days the cultivation when the maximum cell density was achieved. However, the neurites were continuously extended up to 230 μm in adding the polymer even when the cell growth remained steady in Figure 2. In

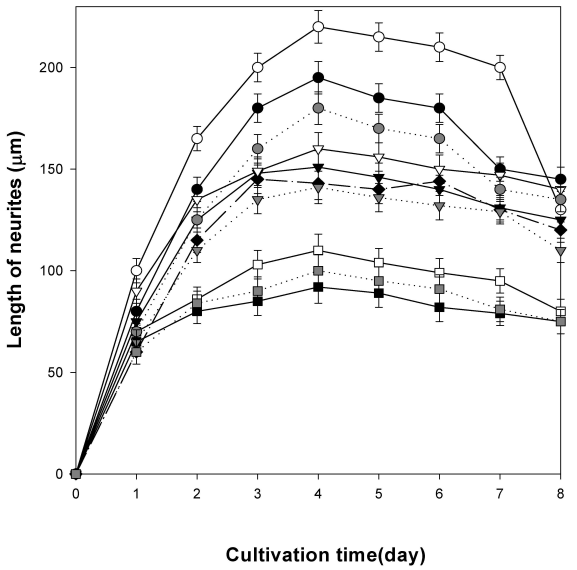

Figure 5. The neurite's extention of PC12 cells in no adding or adding various concentrations of NGF, BDNF and the biopolymer from *H. erinaceus* according to cultivation time: Control(♦): Biopolymer 1.0 mg l^{-1}(●), 0.5 mg l^{-1}(▼), 0.2 mg l^{-1}(■); BDNF 0.1 mg l^{-1}(○), 0.05 mg l^{-1}(▽), 0.02 mg l^{-1}(□); NGF 0.1 mg l^{-1}(gray bullet), 0.05 mg l^{-1}(gray triangle), 0.02 mg l^{-1}(gray square).

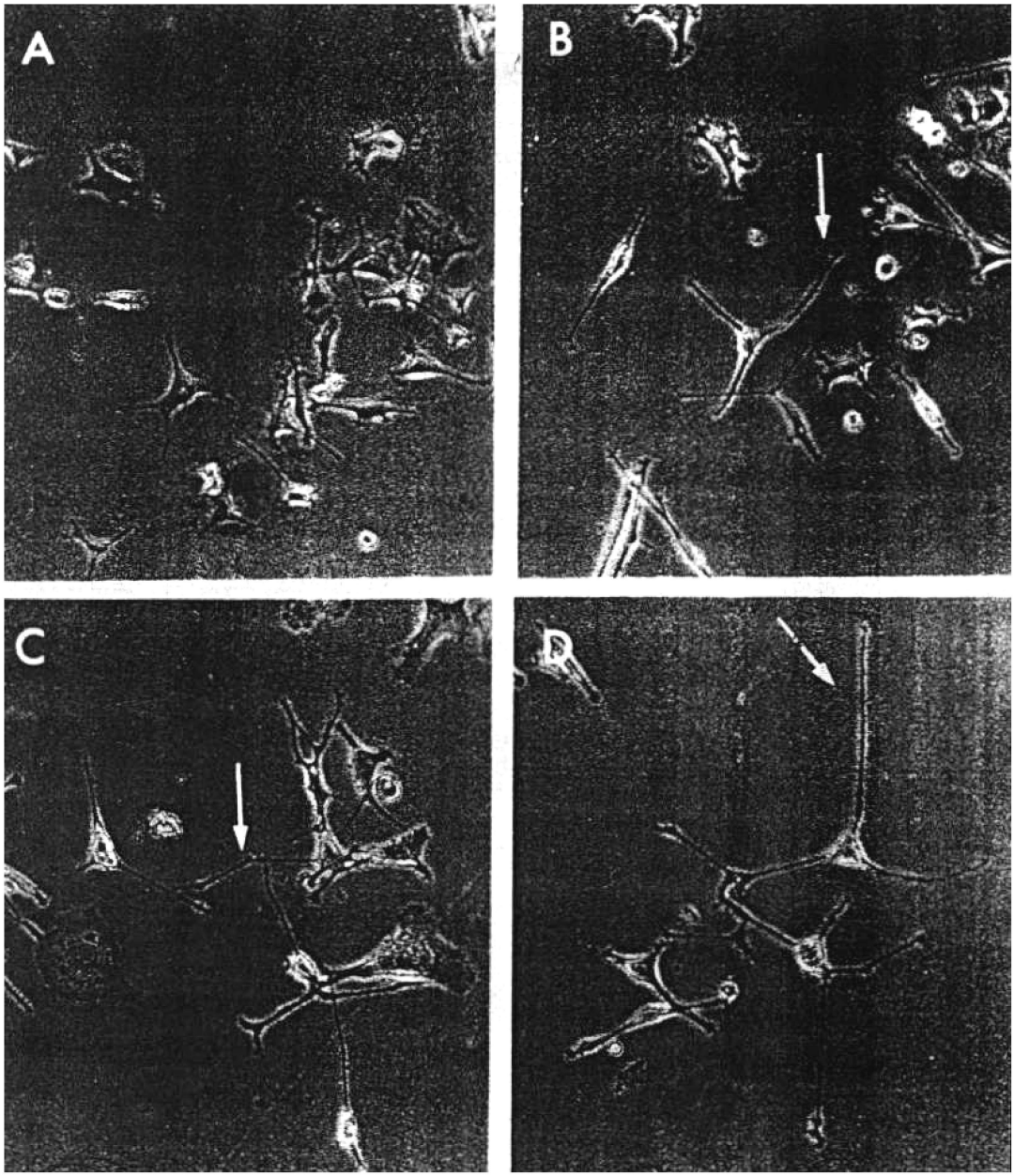

Figure 6. The morphology of PC12 nerve cells in no adding or adding NGF, BDNF and the biopolymer from *H. erinaceus* after 5 days of cultivate. A: Control, B: BDNF, C: NGF, D: Biopolymer.

0.1 (mg l^{-1}) of highest supplementation of two standards, NGF or BNDF, the maximum extension of the neurites was definitely increased compared to the control while the period of extending the neurites was not lengthened. Similar trend was also observed in adding 0.05 (mg l^{-1}) or 0.02 (mg l^{-1}) of lower supplementation, but did not seem to be much effective compared to that in adding lower supplementation of the exo-polymer shown in Figure 1. There was no visible growth of the neurites on the first day, but by the third day, the neurites had grown to about 190 μm in adding the polymer. Then, there were many initiations of the neurites within the cells. Fully developed neurons were observed on the fifth day. The extension of neurites remained relatively steady at latter period of the cultivation in adding the

polymer while decreased in adding growth factors and no addition. The decrease of neurite extension was even greater at latter period of the cultivation for the case of adding NGF or BDNF than for the control. Figure 6 compares the growth and neurite extension of PC12 cells after five days in adding the samples or no addition. Arrows indicate the neurites of PC12 cells. Obvious enhancement of neurite extension was observed in adding exo-polymer (D), compared to the control (A). The length of neurite in adding the polymer seemed to be the longest among others in adding growth factors. At least three or four neurites were maintained within a single cell after six days of the cultivation. It is far developed compared to other reports that more than 10 days were required to develop a similar morphology (D in Figure 6) in growing PC12 cells without supplementing the any growth effectors under normal conditions (Greene and Tischler 1976; deSouza et al. 1995).

Figure 7 shows the number of neurite-bearing cell in adding various concentrations of the samples or standards, respectively. Without adding the samples, the number of neurite-bearing cells was suddenly dropped at latter period of the cultivation when the cell growth was decreased. Its pattern seems to be different from the extension of the neurites shown in Figure 5. There may also be an effect of addition of the sample or standards on increasing the number of neurite-bearing cells when the concentration of the supplementation was increased as shown in Figures 1 and 5. The neurite growth remained constant in adding the biopolymer or nerve growth factors even though the cell growth started to be decreased after 4 days of the cultivation. However, the number of neurite-bearing cells was sensitively affected by the cell growth. The cells that already contain neurites are more likely to make additional neurites than cells without them. In adding the polymer or two growth factors, the number of the cells bearing neurites remained relatively constant when the cell growth was decreased. Among them, the polymer showed the highest effect on enhancing the number of neurite-bearing cells than two other nerve growth factors. There was not much differences in increasing the number of the neurite-bearing cells between two factors. It is also interesting that the polymer seems to work on increasing the number of the neurite-bearing cells after 4 days of the cultivation when the number of the cells greatly dropped without supplementing any growth factors. It implies that the polymer may first work on lengthening the neurites which already

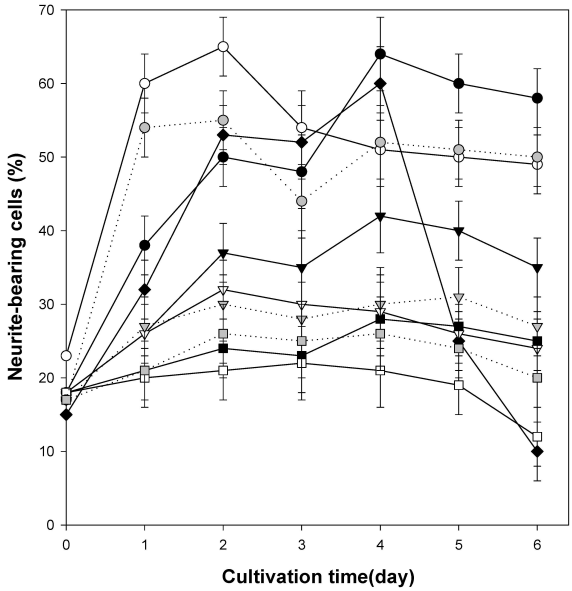

Figure 7. Comparison of the percentage of neurite-bearing cells in no adding or adding various concentrations of NGF, BDNF and the biopolymer according to cultivation time : Control(♦): Biopolymer 1.0 mg l^{-1}(●), 0.5 mg l^{-1}(▼), 0.2 mg l^{-1}(■); BDNF 0.1 mg l^{-1} (○), 0.05 mg l^{-1} (▽), 0.02 mg l^{-1}(□); NGF 0.1 mg l^{-1} (gray bullet), 0.05 mg l^{-1} (gray triangle), 0.02 mg l^{-1} (gray square).

existed in the cell, then enhance the number of cells to initiate the formation of the neurites within the cells.

Table 1 compares the cell growth, number of neurite bearing cells and the growth of neurites in

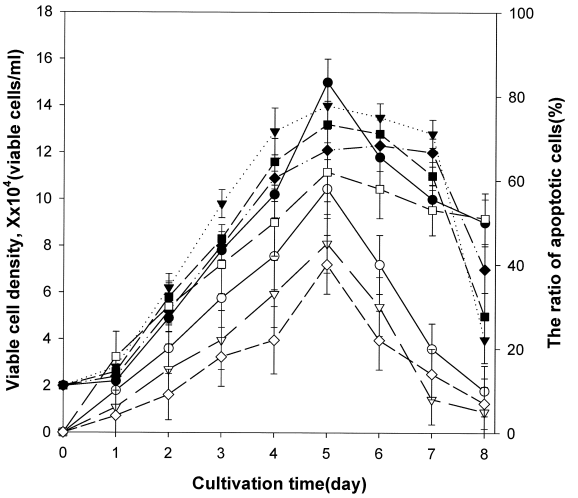

Figure 8. The change of death pattern of PC12 cells in no addition or adding NGF, BDNF and the biopolymer from *H. erinaceus* according to cultivation time: Cell growth, X; control(■), BDNF(▼), NGF(●), Biopolymer(♦): Apoptotic ratio; control(□), BDNF(▽), NGF(○), Biopolymer (◇).

Table 1. Comparison of cell viability, neurite-bearing cells and the extension of neurites in adding several nerve cell growth factors.

Effector	Maximum cell density, X (viable cells ml^{-1})	Cell viability (%)**	Neurite-bearing cell (%)	Max. length of the neurite (μm)
Control	1.11×10^5	53 ± 2.9	51.0 ± 2.4	141 ± 1.4
NGF*	1.15×10^5	57.5 ± 3.1	51.3 ± 1.3	184 ± 2.8
BDNF*	1.21×10^5	73 ± 3.4	60.5 ± 2.1	192 ± 1.1
Biopolymer	1.55×10^5	89 ± 1.9	64.3 ± 1.1	235 ± 1.8

*0.1 (mg l^{-1}) of NGF or BDNF addition
** 8^{th} day of the cultivation.

adding the effectors or no addition (control) after five days cultivation. As shown in previous Figures, the best growth of cells and neurites was observed in 0.8 (mg l^{-1}) of the exo-polymer. In general, all of three effectors positively affected for all cases of cell growth and neurite extension and formation. It was apparent that NGF was not much effective on cell growth and the number of neurite-bearing cells except for the extension of the neurites. It was also found that the polymer can well improve neurite growth rather than cell growth. It possibly implies that the signal for generating neurites within the cell can be possibly induced by adding certain levels of the polymer to affect the nerve cell membrane.

Figure 8 illustrates apoptotic pattern of PC12 cells in adding the samples. The ratio of apoptotic cells continuously increased without adding the samples as the cell growth decreased. However, the ratio of apoptotic to normal cell remained steady and even decreased in adding the effectors while the cell density was dropped at latter period of the cultivation. It was also found that the polymer most effectively prevented the apoptosis of PC12 cells than other growth factors, maintaining less than 20% of apoptotic cells compared to 51% in control. It may tell that the polymer could partially delay the apoptosis of nerve cells and result in increasing the numbers of neurite-bearing cells, which can eventually enhance the growth of nerve cells. Based on these findings, detail *in vivo* mechanism of the exo-polymer from *H. erinaceus* influencing PC12 cells should be further investigated.

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